Microbiology and Immunology

Microbiol Immunol 2015; 59: 114–122
doi: 10.1111/1348-0421.12235

ORIGINAL ARTICLE

Virulence characteristics of Shiga toxin-producing *Escherichia coli* from raw meats and clinical samples

Son Hoang Minh, Etsuko Kimura, Duc Hoang Minh, Ken-ichi Honjoh and Takahisa Miyamoto

Laboratory of Food Hygienic Chemistry, Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University. 6-10-1, Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan

ABSTRACT

Shiga toxin producing *Escherichia coli* (STEC) are dangerous foodborne pathogens. Foods are considered as important sources for STEC infection in human. In this study, STEC contamination of raw meats was investigated and the virulence factors of 120 clinical STEC strains characterized. STEC was detected in 4.4% of tested samples. Among 25 STEC strains from meats, five strains (20%) were positive for the *eae* gene, which encodes intimin, an important binding protein of pathogenic STEC. The remaining strains (80%) were *eae*-negative. However, 28% of them possessed the *saa* gene, which encodes STEC agglutinating adhesin. The *ehxA* gene encoding for enterohemolysin was found in 75% of the meat strains and the *subAB* gene, the product is of which subtilase cytotoxin, was found in 32% of these strains. The stx_{2a} gene, a subtype of Shiga toxin gene (*stx*), was the most prevalent subtype among the identified meat STEC bacteria. None of the meat STEC was O157:H7 serotype. Nevertheless, 92% of them produced Shiga toxin (Stx). Among 120 clinical STEC strains, 30% and 70% strains harbored single and multiple *stx* subtypes, respectively. Most clinical STEC bacteria possessed *eae* (90.8%) and *ehxA* (96.7%) genes and 92.5% of them showed Stx productivity. Our study shows that some raw meat samples contain non-O157 STEC bacteria and some strains have virulence factors similar to those of clinical strains.

Key words *Escherichia coli* O157:H7, STEC, stx-subtypes, Verotoxin.

Shiga toxin-producing *Escherichia coli*, also known as verotoxin-producing *E. coli*, are dangerous pathogens that can cause diseases in human ranging from mild diarrhea to serious conditions such as HC, HUS and death (1). STEC serotype O157:H7 is frequently associated with outbreaks or sporadic cases of illness; however, non-O157 STEC strains have also been found to cause HC or HUS (2). The STEC strains are characterized by the ability to produce one or more Stx. The Stx family consists of two groups designated as Stx1 and Stx2 (3). Stx1 is a highly conserved group consisting of three variants, Stx1a, Stx1c and Stx1d (4, 5), whereas Stx2 is a diverse group composed of seven distinct variants, namely Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g (6). Among these variants, some toxins are frequently found in the STEC strains that can cause severe illness in human (7, 8). Production of Stx alone is usually insufficient for STEC strains to cause disease. Studies have shown that accessory virulence factors such as intimin, enterohemolysin, STEC auto-agglutinating adhesion and subAB, which are encoded by *eae*, *ehxA*, *saa* and *subAB* genes, respectively, are usually needed for STEC to be pathogenic (3, 9).

Correspondence
Takahisa Miyamoto, Laboratory of Food Hygienic Chemistry, Department of Bioscience and Biotechnology, Kyushu University, 6-10-1, Hakozaki, Higashi-ku, Fukuoka-shi, 812-8581, Japan. Tel./fax: +81 92 6429 3023; email: tmiyamot@agr.kyushu-u.ac.jp

Received 11 December 2014; revised 26 January 2015; accepted 27 January 2015.

List of Abbreviations: *eae*, intimin protein encoding gene; EC, *Escherichia coli*; *ehxA*, enterohemolysin encoding gene; HC, hemorrhagic colitis; HUS, hemolytic uremic syndrome; H−, H-antigen untypeable; OUT, O-antigen untypeable; RPLA, reverse passive latex agglutination; *saa*, Shiga toxin producing *E. coli* agglutinating adhesin; STEC, Shiga toxin producing *E. coli*; Stx, Shiga toxin; *stx*, Shiga toxin gene; subAB, subtilase cytotoxin; *subAB*, subtilase cytotoxin gene; VTEC, Verotoxin-producing *E. coli*.

Shiga toxin-producing *Escherichia coli* infections in humans occur mainly through ingestion of contaminated foods (10). Cattle have been identified as the main reservoir of STEC (11). Contamination of carcasses with STEC may occur during slaughtering and processing. Thus, raw meats, especially ground meats, are at high risk of STEC contamination. Several investigations of STEC presence in raw meats have been carried out in the past few years (12–15). However, to enable development of effective STEC control strategies, better understanding of their prevalence and virulence gene profiles is still needed. The aim of this work was to determine the presence and characteristics of STEC from raw meats and characterize the main virulence factors of clinical STEC strains that had previously been isolated from ill humans for reference purposes.

MATERIALS AND METHODS

Sampling and culture enrichment

From 2009 to 2010, 293 raw-ground meat samples, including 97 of beef, 26 of pork, 23 of chicken and 147 of mixed beef–pork, were purchased from three retail stores near the Hakozaki campus of Kyushu University, Fukuoka-Japan. The samples were kept in a cool box while being transported to a laboratory and processed immediately upon arrival. Each sample (25 g) was homogenized with 225 mL sterilized modified EC broth containing 20 mg/L of novobiocin (mEC + n) (Eiken Chemical, Tokyo, Japan) by Pulsifier (Microgen, Surrey, UK) for 15 s. The homogenates were then incubated overnight at 42 °C.

Detection of *stx* genes and isolation of STEC from meats

For the detection of *stx* genes in raw meat samples, bacterial cells (1 mL) were harvested from the overnight cultures and used for DNA extraction using a DNeasy tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA samples were used as templates for PCR targeting of stx_1 or/and stx_2 genes of STEC bacteria using EVT and EVS primer sets, respectively (16). The PCR was carried out in a 25 μL reaction mixture consisting of 2.5 μL 10× PCR buffer (Takara Bio, Kyoto, Japan), 0.5 μL of 2.5 mM deoxynucleotide triphosphate mixture, 0.25 μL of 0.2 μM each primer (Takara Bio), 0.125 μL of Taq DNA polymerase (Takara Bio), 20.375 μL sterilized distilled H_2O and 1.0 μL template DNA. The genomic DNA from *E. coli* O157:H7 harboring both stx_1 and stx_2 genes and K-12 strains were used as positive and negative controls, respectively. The thermal cycling included an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The amplicons were examined by agarose gel electrophoresis and viewed by using gel photographing system (Cosmos Biotech, Tokyo, Japan).

To isolate STEC bacteria from meat samples positive for *stx* genes, bacterial selective culture was incorporated into a colony-PCR method. Five milliliters of the corresponding enrichment broth were inoculated into 45 mL MacConkey broth (Merck, Darmstadt, Germany) and incubated at 37 °C for 13 hr. After incubation, 1 mL of the culture was withdrawn and serially diluted with PBS. The dilution (100 μL) was spread over CHROMagar O157 (CHROMagar, Paris, France) supplemented with cefixime (0.05 mg/L) and tellurite (2.5 mg/L) and incubated overnight at 37 °C. Plates with approximately 300 colonies were chosen and up to 30 colonies per sample were picked up for colony PCR using the same primers as in the detection step described above. The colonies that were positive for *stx* genes were re-streaked on the same agar, confirmed by the same PCR and stored in tryptic soy broth (Becton Dickinson, Oxford, UK) containing 25% (vol/vol) glycerol at −80 °C for later use.

Characterization of isolated and clinical STEC strains

A RiboPrinter System (DuPont Qualicon, Boston, MA, USA) was used according to the manufacturer's instructions to identify the species of the isolated bacteria. The identified bacterial strains were then subjected to serotyping. The O-antigen groups of the STEC from meats were determined by using an O-antisera kit (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions. The O-antigen identifiable STEC strains were then tested for the presence of H-antigen type by using an H-antisera kit (Denka Seiken) according to the manufacturer's instructions.

To compare the virulence profile between STEC strains isolated from meats and humans, 120 clinical STEC strains (33 non-O157 and 87 O157 serotype) were subjected to characterization testing. The clinical bacteria had been isolated from subjects with food poisoning by the Fukuoka City Institute for Hygiene and Environment, Fukuoka City, Japan during human health monitoring programs from 1997 to 2006 and their characteristics had not been fully determined before this study. The virulence genes of the STEC strains were determined by PCR. Briefly, bacteria were cultured in 5 mL tryptic soy broth at 37 °C overnight. The culture broth (1 mL) was used for DNA extraction as described above. The obtained DNA samples were used as

templates for PCR. The presence of *stx* subtypes was determined by PCR as described by He *et al.* (17). The *eae* gene was detected by using eae-F: 5′-GGCGATTACGC-GAAAGATAC-3′/ eae-R: 5′-GGCCTGCAACTGTGAC-GAAG-3′ primers designed in our laboratory. For detection of the *ehxA* and *saa* genes, primers developed by Quinones *et al.* (18) were employed. The PCR for *eae, ehxA* and *saa* genes was carried out as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles consist of 94 °C for 45 s, 55 °C for 60 s, 72 °C for 60 s and final extension at 72 °C for 10 min. For detection of the *subAB* gene, the primers developed by Funk *et al.* (19) were used.

Production of Shiga toxin by the meat and clinical STEC strains was determined by using a VTEC RPLA kit (Denka Seiken). Briefly, the STEC strains were cultured in brain heart infusion agar (Oxoid, Cambridge, UK) overnight at 37 °C. The bacteria were harvested and suspended in 1 mL of PBS containing 5000 units/mL polymyxin B (Naclai Chemicals, Tokyo, Japan). The bacterial suspensions were incubated at 37 °C for 30 min and centrifuged at 900 *g* for 15 min. The cell pellets were then discarded. Shiga toxin in the supernatant was detected using Verotoxin-RPLA kit according to the manufacturer's instructions. Titers of Shiga toxin were defined as the reciprocal of the highest dilution that gave a positive reaction in the VTEC-RPLA assay. Titers less than 2 were interpreted as negative.

RESULTS

Detection of *stx* genes and isolation of STEC from raw meats

According to PCR detection of *stx* genes, all four meat types were positive after enrichment culture. The details of the PCR are shown in Table 1. *stx* genes were detected in 56 of the 293 meat samples examined (19.1%). The stx_2 gene was more frequently positive (14.7%) than the stx_1 gene (6.8%). Both stx_1 and stx_2 genes were positive in seven samples (2.4%). Regarding meat types, the *stx*-positive rates of beef, pork, chicken and beef–pork were 19.6%, 23.1%, 17.4% and 18.4%, respectively. There was no significant difference between samples in the *stx*-positive rate ($P > 0.05$).

All meat samples positive for *stx* genes were subjected to STEC isolation, the results are shown in Table 1. STEC bacteria were recovered from 13 of the 56 samples analyzed (23.2%), accounting for 13/293 (4.4%) of all the samples. More than one STEC isolate was sometimes recovered from one sample (data not shown); thus, there were 25 isolates all together. The rates of STEC isolation were 7.2%, 3.8%, 0% and 3.4% for beef, pork, chicken and beef–pork, respectively (Table 1). There was no significant difference between beef and pork samples in the rate of STEC isolation ($P > 0.05$). The PCR results showed that 5/25 (20%) and 20/25 (80%) isolates were positive for stx_1 and stx_2 genes, respectively (Table 1). None of them contained both genes. The stx_1-harboring STEC were isolated from beef samples only, whereas stx_2-harboring STEC were recovered from beef, pork and beef–pork samples (Table 1).

Ribotyping and serotyping of STEC from raw meats

The Ripoprinter system verified all 25 meat STEC isolates as *E. coli* (data not shown). The O-antigens of 8/25 isolates (32%) were determined; these belonged to four serogroups, namely O1, O6, O74 and O91. None of them were typed as O157 serogroup (Table 2). The remaining 17/25 isolates (68%) had O-antigen untypeable (designed as OUT). H-antigen tests on the eight STEC isolates whose O-antigens had been were identified showed that they consisted of three H-groups, namely H21, H9, and H− (H-antigen non-motile) (Table 2).

Characterization of virulence genes of meat and clinical STEC

The virulence characteristics of 25 meat STEC strains are listed in Table 2. Subtyping tests on the five stx_1-positive

Table 1. Detection of *stx* genes and isolation of STEC bacteria from raw meats

Meat type	No. of samples analyzed	Detection of *stx* genes				Isolation of STEC bacteria			
		No. of positive samples (%)	No. of samples positive for gene (%)			No. of positive samples (%)	No. of STEC isolate positive for gene		
			stx_1	stx_2	stx_1 and stx_2		stx_1	stx_2	stx_1 and stx_2
Beef	97	19 (19.6)	9 (9.3)	13 (14.4)	3 (3.1)	7 (7.2)	5	6	0
Pork	26	6 (23.1)	1 (3.8)	5 (19.2)	0	1 (3.8)	0	4	0
Chicken	23	4 (17.4)	2 (8.7)	3 (13.0)	1 (4.3)	0	0	0	0
Beef–pork	147	27 (18.4)	8 (5.4)	22 (15.0)	3 (2.0)	5 (3.4)	0	10	0
Total	293	56 (19.1)	20 (6.8)	43 (14.7)	7 (2.4)	13 (4.4)	5	20	0

Table 2. Virulence characteristics of STEC strains isolated from raw meats

			Accessory virulence gene				Shiga toxin titer by RPLA	
Meat type	Serotype	*stx* gene subtype (no. of strains)	*eae*	*ehxA*	*saa*	*subAB*	Stx1	Stx2
Beef	OUT:Hnd	1a (2)	+	+	−	−	+	
		1a (3)	+	+	−	−	+	
		2a (1)	−	+	+	+		+
		2a, 2d (1)	−	+	+	+		+
		2a, 2d (2)	−	+	+	+		+
		2a, 2b (1)	−	+	−	+		+
		2a (1)	−	+	+	+		+
Pork	OUT:Hnd	2e (2)	−	−	−	−		+
		2b, 2e (1)	−	−	−	−		+
		2e (1)	−	−	−	−		+
Beef–pork	O1:H21	2a (2)	−	−	−	−		+
	O6:H9	2e (1)	−	−	−	−		+
	O74:H−	2g (2)	−	+	−	−		− [2]
	O6:H−	2e (1)	−	−	−	−		+
	O91:H21	2d (2)	−	−	−	−		+
	OUT:Hnd	2a (2)	−	+	+	+		+

Hnd, H-antigen not determined.
Square brackets denote number of strains negative for Stx.

strains showed that they all had stx_{1a} gene only. None of them possessed other subtypes such as stx_{1c} or stx_{1d}. stx_{2a} was the most prevalent variant, being found in 10/25 strains(40%), followed by stx_{2e} (24%), stx_{2d} (20%), stx_{2b} (8%) and stx_{2g} (8%). Three stx_2 subtype combinations were identified in the stx_2-positive group (Table 2). Characterizations of accessory virulence genes from meat STEC showed that five stx_{1a}-containing strains were also positive for *eae* gene, whereas all 20 stx_2-positive isolates were negative for this gene. The *ehxA*, *saa* and *subAB* genes were found in 15/25 (75%), 7/25 (28%) and 8/25 (32%) STEC strains, respectively (Table 2).

Of the 120 clinical STEC strains, 19 (15.8%), 32 (27.7%), and 69 (57.5%) strains were positive for stx_1, stx_2, and stx_1 and stx_2 genes, respectively. *stx*-subtyping showed that 36/120 strains (30%) had only a single *stx* subtype, either stx_{1a}, stx_{2a}, stx_{2b} or stx_{2c}, whereas the remaining 84/120 strains (70%) had combinations of four, three and two *stx* subtypes (Table 3). Among the stx_1-positive strains, all were subtyped as stx_{1a} variant; six strains additionally had the stx_{1c} gene. In the stx_2-positive group, stx_{2a} was the most prevalent subtype, being found in 77/101 strains (76.2%), followed by stx_{2b} (41.5%), stx_{2c} (31.6%) and stx_{2d} (1.9%). None of the clinical STEC strains contained stx_{1b}, stx_{2e}, stx_{2f} or stx_{2g} genes (Table 3). Additional virulence gene characterization of clinical STEC showed that 109/120 (90.8%) and 116/120 (96.7%) strains had *eae* and *ehxA* genes, respectively. All 11 strains (0.09%) that were negative for *eae* gene belonged to the non-O157 group. Only 1/4 *ehxA*-negative strains were STEC O157, the other three strains were non-O157. There were 3/11 *eae*-negative STEC strains (27.2%) that were positive for *subAB* gene. Of these three *subAB*-positive STEC, two strains contained *saa* gene and the remaining STEC serotype O128:H2 was negative for this gene (Table 3).

Shiga toxin production

Shiga toxin production by meat and clinical STEC is shown in Tables 2 and 3, respectively. Among 25 meat STEC strains, 23 strains (92%) produced varying amounts of Stx toxin, the titers ranging from 2 to 2048. Only two strains of serotype O74:H− that had stx_{2g} gene did not produce any toxins. Of the 120 clinical STEC bacteria, 9 strains (7.5%) did not produce toxins, whereas the remaining strains (92.5%) produced varying titers of Stx. Among the nine clinical STEC strains negative for Stx, eight strains (88.8%) were O157:H7 serotype and the other was O128:H- STEC.

DISCUSSION

Consumption of food contaminated with STEC bacteria can cause infection in humans. However, detection of STEC in foods is not always easy because of several factors, including the following: (i) STEC bacteria are usually present in small numbers compared with the background micro-flora in food samples; (ii) the

Table 3. Virulence characteristics of clinical STEC strains

		Accessory virulence gene				Shiga toxin titer by RPLA	
Serotype	*stx* gene subtype (no. of strains)	*eae*	*ehxA*	*saa*	*subAB*	Stx1	Stx2
O103:H2	1a (1)	+	+	−	−	+	
	1a, 1c (1)	+	+	−	−	+	
O103:H−	1a (1)	+	+	−	−	+	
O111	1a, 2a, 2b (1)	+	+	−	−	+	+
O111:H-	1a (2)	+	+	−	−	+	
O114:H19	2a (1)	+	+	−	−		+
O119:H-	1a (2)	−	+	−	−	+	
O121:H19	2a (1)	+	+	−	−		+
O128:H-	2a (1)	−	+	−	−		−[1]
O128:H2	1a, 1c, 2b (1)	−	+	−	+	+	+
O152:H−	1a (1)	+	+	−	−	+	
O26	1a (4)	+	+	−	−	+	
	1a, 2a (1)	+	+	−	−	+	+
	1a, 1c, 2a (1)	+	+	−	−	+	+
O26:H11	1a (1)	+	+	−	−	+	
	1a, 2a (1)	+	+	−	−	+	+
	1a, 1c, 2a (1)	+	+	−	−	+	+
	1a, 1c, 2a, 2b (1)	+	+	−	−	+	+
	1a, 2a, 2b (1)	+	−	−	−	+	+
	1a, 1c, 2a (1)	+	+	−	−	+	+
O6:H−	2a (1)	−	+	+	+		+
O74:H−	2b, 2d (1)	−	+	−	−		+
O8:H19	2d (1)	−	−	−	−		+
O91:H−	1a (2)	−	+	−	−	+	
OUT	1a (1)	−	−	−	−	+	
	1a (1)	−	+	−	−	+	
OUT:H−	2a (1)	−	+	+	+		+
O157	2a (1)	+	−	−	−		+
	2c (1)	+	+	−	−		+
	2a (4)	+	+	−	−		+
	2a, 2b, 2c (2)	+	+	−	−		+
	2a, 2c (1)	+	+	−	−		+
	1a, 2a (26)	+	+	−	−	+	+
	1a, 2a, 2b, 2c (5)	+	+	−	−	+	+
	1a, 2a, 2b (6)	+	+	−	−	+	+
O157:H−	2a, 2c (1)	+	+	−	−		+
	1a, 2b, 2c (2)	+	+	−	−	+	+
	1a, 2a, 2b, 2d (1)	+	+	−	−	+	+
O157:H7	1a (1)	+	+	−	−	+	
	2b, 2c (4)	+	+	−	−		[2]
	2a, 2b, 2c (1)	+	+	−	−		+
	2c (3)	+	+	−	−		+
	2a (2)	+	+	−	−		+
	2a, 2b (1)	+	+	−	−		+
	2a, 2c (3)	+	+	−	−		+
	1a, 2a (6)	+	+	−	−	+	+
	1a, 2b (5)	+	+	−	−	− [1]	− [3]
	2b (1)	+	+	−	−		[1]
	1a, 2a, 2b, 2c (4)	+	+	−	−	+	[1]
	1a (1)	+	+	−	−	+	
	1a, 2b, 2c (5)	+	+	−	−	+	+

Square brackets denote number of strains negative for Shiga toxin (Stx).

detection methods require a specific protocol that can discriminate STEC from nonpathogenic *E. coli;* and (iii) STEC strains that have only partial virulence profiles are also present in these samples. In this study, raw meats were screened for the presence of *stx*-genes. Contamination of these samples by STEC bacteria was confirmed by isolation of viable cells. The virulence characteristics of STEC isolates from meats were then analyzed and compared with those from humans to assess the possible risk.

The PCR revealed that all four meat types were positive for *stx* genes (Table 1), suggesting that these meats were contaminated with STEC bacteria. However, viable STEC strains were isolated from only 13/56 *stx*-positive samples (23.2%), accounting for 4.4% of all samples. A failure to recover STEC from samples positive for *stx* genes has also occurred in previous studies (13, 15). Possible reasons for this include the presence of very small numbers of STEC cells that could not be isolated on the selective agar and the presence of *stx*-carrying phages in the meat samples (20, 21). The overall prevalence of STEC in meats in our study was 4.4%, which is higher than the 1.75% reported by Fantelli *et al.* (22), but lower than the 5.2% identified by Ju *et al.* (15) and the 21.7% reported by Barlow *et al.* (23). In our opinion, these discrepancies may originate from differences in either the studies' locations or the testing methods used in each analysis.

Shiga toxin-producing *Escherichia coli* are heterogeneous, carrying various virulence genes, not all of which are equally pathogenic for humans (24, 25). Thus, characterization of the virulence genes of STEC is important for risk estimation. We found that 20% and 70% of STEC isolates from meats and clinical samples, respectively, contained multiple *stx* subtypes. This finding implies that most clinical STEC strains have much more complex *stx* subtypes than meat strains.

In meat STEC, the stx_1 gene is reportedly less prevalent than the stx_2 gene and most stx_1-positive strains (90%) are subtyped as stx_{1a} variant (15). We observed a similar trend in our study: only 20% of strains from meats were positive for stx_1 and all of them were subtyped as stx_{1a} subtype. Even though stx_{1a}-positive strains were not abundant in our meat STEC isolates, the fact that they all additionally carried *eae* and *ehxA* genes, which are considered critical virulence markers for STEC's pathogenesis (3) and that these were also found in 90.8% and 96.7% of clinical STEC strains, respectively (Table 3), persuades us that these five meat STEC strains are pathogenic for humans.

Among the stx_2 subtypes, stx_{2a} has been considered a typical virulence gene that is frequently detected in STEC strains causing HC or HUS (25). We identified this subtype regularly in our study, 76.2% of clinical STEC strains being positive for stx_2 gene subtyping as stx_{2a}. Several studies have reported finding the stx_{2a} gene not only in clinical strains but also in those from fresh produce (56%) (26) and natural cheese (40%) (27). However, data on the presence of stx_{2a} gene in STEC strains isolated from raw meats are limited. In the USA, it was found in 65.7% of STEC strains isolated from ground beef (13). Our finding that 40% of meat STEC isolates was positive for stx_{2a} is comparable with the above reported data and suggests that stx_{2a} gene probably circulates widely in STEC strains from raw meats. Despite being negative for *eae* gene, 7/10 stx_{2a}-positive strains (70%) were additionally positive for both *ehxA* and *saa* genes, which are thought to contribute to STEC's pathogenicity by acting as a cytotoxin (28) and facilitating attachment to intestinal cells (29), respectively. Considering the contribution of these two genes to STEC as well as the virulence gene recognized in two clinical STEC (O6:H−; OUT:H−) (Table 3), it can be assumed that these seven strains of *eae*-negative STEC isolates can cause human illness.

In contrast with the stx_{2a} gene, stx_{2b} alone was not frequently detected from STEC strains causing HC or HUS (30). stx_{2b} has been seen in STEC strains from deer, goat and sheep meats (31). In our meat STEC strains, we found stx_{2b} in association with other stx_2 variants in 8% of samples. This rate suggests that stx_{2b} may occur infrequently among STEC strains from pork and beef. These findings are supported by the study of Ju *et al.* (15), who recently performed a study that was almost identical to ours and also found the stx_{2b} gene in only 3% of STEC strains from ground meats.

Of the Stx-variants, Stx2d has a unique working mechanism whereby its toxicity can be increased a thousand-fold following activation of elastase in the intestinal mucus (32). Ju *et al.* reported that in the USA, 28.1% of STEC strains from ground meats contained stx_{2d} gene (15). Because we also analyzed STEC strains from ground meats and observed the presence of stx_{2d} gene in 20% of these bacteria, it can be postulated that meat STEC strains can bear the toxic *stx* subtype like the stx_{2d} gene. Furthermore, among the five stx_{2d}-positive STEC from meats, two strains of serotype O91:H21 that were stx_{2d}-positive and *eae*-negative should be treated carefully because STEC serotype O91:H21, which harbors the same virulence gene designations, has been found to cause human illness (33, 34).

Research has shown that the stx_{2e} gene is identified mainly in pig or pork STEC isolates (15, 31, 35). It was the second most common stx_2 variant in our meat STEC isolates (24%). The high prevalence of stx_{2e} gene in meat STEC may be explained by the fact that 173/293 meat

samples (59%) analyzed in the present study consisted of only pork (26 samples) or pork mixed with beef (147 samples). Logically, the rate of successful isolation of stx_{2e}-positive STEC strains would be high in samples of pork origin. Despite the fact that STEC strains with the stx_{2e} gene were quite common among STEC strains from meats, they were all negative for *eae, ehxA, saa* or *subAB* genes. Regarding the virulence gene composition of these stx_{2e}-positive STEC strains, they seem to be confer low risk or be harmless for humans.

Since the first description of the stx_{2g} gene in STEC strains from cattle by Leung *et al.* (36), some studies have recorded low frequencies of this gene in STEC strains from various sources such as game meats and ready-to-eat meat products (12.5%) (37), fresh produce (2.2%) (26) and other food such as wildlife meat, raw milk and raw-milk cheese (2.7%) (12). In our study, we also detected the stx_{2g} gene at a low rate (8%) in meat STEC isolates. Prager *at al.* reported that stx_{2g}-harboring STEC strains were negative for *eae* and *ehxA* genes in their study as well as in others (38). Thus, it is probable that STEC strains containing both stx_{2g} and *ehxA* genes like ours are rarely found. Interestingly, the STEC strains positive for stx_{2g} in our studies were serotyped as O74:H−. To the best of our knowledge, STEC O74:H− containing stx_{2g} gene has not been previously reported. Although the pathogenic potentials of the stx_{2g}-positive STEC strains for humans have not yet been evaluated (38), our stx_{2g}-positive strains containing *ehxA* gene deserve attention.

The *subAB* gene is usually detected in *eae*-negative STEC (9). SubAB causes some pathogenic characteristics typical of Stx-induced HUS *in vivo* (39). In the present study, we found that *eae*-negative STEC strains from meats and humans were positive for the *subAB* gene at 40% and 27.2%, respectively (Tables 2 and 3). Even though Stx is a major virulence factor of STEC bacteria, the fact that subAB can induce HUS by damaging human microvascular cells (40) and directly participates in causing diarrhea in children (41), suggests that the presence of *subAB* gene in STEC strains may increase their risk for humans.

The most predominant serotype associated with HC and HUS are STEC O157:H7 and their presence in meats has been reported for some years (11, 21, 42, 43). However, in the current study, we detected no STEC O157:H7 serotypes in raw meats. This is probably attributable to the low prevalence of O157:H7 serotype in Japan, as recently announced in a report of the national food surveillance system (44). The absence of O157:H7 in our meat STEC probably reflects the current true prevalence of this serotype; however, it definitely should not be considered a safe marker for STEC from raw meats because we recovered other serogroups such as O1, O6, and O91 that are also associated with HC and/or HUS (45).

Among culture supernatants of 25 meat STEC isolates, 23 strains (92%) were positive for Stx. Two strains of serotype O74:H− that were positive for stx_{2g} and *ehxA* genes were negative for Stx. The lack of Stx in these two strains is not surprising because stx_{2g}-positive STEC strains not producing Stx have also been identified in some studies, these findings being attributed to the presence of stx_{2g} pseudogene or mutations (12, 38, 46). Eight STEC O157:H7 strains from humans were negative for Stx production by VTEC-RPLA assay (Table 3). Furthermore, even after induction by mitomycin, five of these eight strains did not produce Stx2 (data not shown). Currently, there is little information about STEC O157 that are negative for Stx production. Nonetheless, these findings probably relates to mechanisms such as the small amounts of Stx released in the culture being undetectable by kit, Stx not being released into culture because of problems of secretion; or STEC bacteria carrying defective phage DNA (47, 48).

In summary, this study was designed to explore the prevalence and virulence characteristics of STEC bacteria isolated from raw meats and to compare them with the virulence traits of STEC from clinical samples. We found that 4.4% of raw meat samples were contaminated with non-O157 STEC bacteria. Some of STEC strains isolated from meat had virulence factors that were similar to those of the strains isolated from human illness. Compared with the meat STEC strains, those from humans usually had more complicated virulent gene compositions.

DISCLOSURE

There are no conflicts of interest to declare.

REFERENCES

1. Tarr P.I., Gordon C.A., Chandler W.L. (2005) Shiga-toxin-producing *Escherichia coli* and haemolytic uraemic syndrome. *Lancet* **365**: 1073–86.
2. Smith J.L., Fratamico P.M., Gunther IV N.W. (2014) Shiga toxin-producing *Escherichia coli*. In Sima S., Geoffrey M.G. eds. Advances in Applied Microbiology. Burlington, US: Academic Press pp. 145–97.
3. Paton J.C., Paton A.W. (1998) Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin Microbiol Rev* **11**: 450–79.
4. Burk C., Dietrich R., Acar G., Moravek M., Bulte M., Martlbauer E. (2003) Identification and characterization of a new variant of Shiga toxin 1 in *Escherichia coli* ONT:H19 of bovine origin. *J Clin Microbiol* **41**: 2106–12.
5. Zhang W., Bielaszewska M., Kuczius T., Karch H. (2002) Identification, characterization, and distribution of a Shiga toxin

1 gene variant (stx1c) in *Escherichia coli* strains isolated from humans. *J Clin Microbiol* **40**: 1441–6.
6. He X., Quinones B., McMahon S., Mandrell R.E. (2012) A single-step purification and molecular characterization of functional Shiga toxin 2 variants from pathogenic *Escherichia coli*. *Toxins* **4**: 487–504.
7. Jelacic J.K., Damrow T., Chen G.S., Jelacic S., Bielaszewska M., Ciol M., Carvalho H.M., Melton-Celsa A.R., O'Brien A.D., Tarr P.I. (2003) Shiga toxin-producing *Escherichia coli* in Montana: bacterial genotypes and clinical profiles. *J Infect Dis* **188**: 719–29.
8. Orth D., Grif K., Khan A.B., Naim A., Dierich M.P., Würzner R. (2007) The Shiga toxin genotype rather than the amount of Shiga toxin or the cytotoxicity of Shiga toxin *in vitro* correlates with the appearance of the hemolytic uremic syndrome. *Diagn Microbiol Infect Dis* **59**: 235–42.
9. Paton A.W., Srimanote P., Talbot U.M., Wang H., Paton J.C. (2004) A new family of potent AB(5) cytotoxins produced by Shiga toxigenic *Escherichia coli*. *J Exp Med* **200**: 35–46.
10. Erickson M.C., Doyle M.P. (2007) Food as a vehicle for transmission of Shiga toxin-producing *Escherichia coli*. *J Food Prot* **70**: 2426–49.
11. Hussein H.S. (2007) Prevalence and pathogenicity of Shiga toxin-producing *Escherichia coli* in beef cattle and their products. *J Anim Sci* **85**: E63–72.
12. Beutin L., Miko A., Krause G., Pries K., Haby S., Steege K., Albrecht N. (2007) Identification of human-pathogenic strains of Shiga toxin-producing Escherichia coli from food by a combination of serotyping and molecular typing of Shiga toxin genes. *Appl Environ Microbiol* **73**: 4769–75.
13. Bosilevac J.M., Koohmaraie M. (2011) Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* isolates from commercial ground beef in the United States. *Appl Environ Microbiol* **77**: 2103–12.
14. Xia X., Meng J., McDermott P.F., Ayers S., Blickenstaff K., Tran T.T., Abbott J., Zheng J., Zhao S. (2010) Presence and characterization of Shiga toxin-producing *Escherichia coli* and other potentially diarrheagenic *E. coli* strains in retail meats. *Appl Environ Microbiol* **76**: 1709–17.
15. Ju W., Shen J., Li Y., Toro M.A., Zhao S., Ayers S., Najjar M.B., Meng J. (2012) Non-O157 Shiga toxin-producing *Escherichia coli* in retail ground beef and pork in the Washington D.C. area. *Food Microbiol* **32**: 371–7.
16. Li R., Harada T., Honjoh K., Miyamoto T. (2010) Phylogenetic analysis and Shiga toxin production profiling of Shiga toxin-producing/enterohemorrhagic *Escherichia coli* clinical isolates. *Microb Pathog* **49**: 246–51.
17. He X., Quinones B., McMahon S., Mandrell R.E. (2012) A single-step purification and molecular characterization of functional Shiga toxin 2 variants from pathogenic *Escherichia coli*. *Toxins (Basel)* **4**: 487–504.
18. Quinones B., Swimley M.S., Narm K.E., Patel R.N., Cooley M.B., Mandrell R.E. (2012) O-antigen and virulence profiling of shiga toxin-producing *Escherichia coli* by a rapid and cost-effective DNA microarray colorimetric method. *Front Cell Infect Microbiol* **2**: 61.
19. Funk J., Stoeber H., Hauser E., Schmidt H. (2013) Molecular analysis of subtilase cytotoxin genes of food-borne Shiga toxin-producing *Escherichia coli* reveals a new allelic subAB variant. *BMC Microbiol* **13**: 230.
20. Pradel N., Livrelli V., De Champs C., Palcoux J., Reynaud A., Scheutz F., Sirot J., Joly B., Forestier C. (2000) Prevalence and characterization of Shiga toxin-producing *Escherichia coli* isolated from cattle, food, and children during a one-year prospective study in France. *J Clin Microbiol* **38**: 1023–31.
21. Samadpour M., Barbour M.W., Nguyen T., Cao T.M., Buck F., Depavia G.A., Mazengia E., Yang P., Alfi D., Lopes M., Stopforth J.D. (2006) Incidence of enterohemorrhagic *Escherichia coli*, *Escherichia coli* O157, *Salmonella*, and *Listeria monocytogenes* in retail fresh ground beef, sprouts, and mushrooms. *J Food Prot* **69**: 441–3.
22. Fantelli K., Stephan R. (2001) Prevalence and characteristics of Shigatoxin-producing *Escherichia coli* and *Listeria monocytogenes* strains isolated from minced meat in Switzerland. *Int J Food Microbiol* **70**: 63–9.
23. Barlow R.S., Gobius K.S., Desmarchelier P.M. (2006) Shiga toxin-producing *Escherichia coli* in ground beef and lamb cuts: Results of a one-year study. *Int J Food Microbiol* **111**: 1–5.
24. Friedrich A.W., Bielaszewska M., Zhang W.L., Pulz M., Kuczius T., Ammon A., Karch H. (2002) *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *J Infect Dis* **185**: 74–84.
25. Scheutz F., Teel L.D., Beutin L., Piérard D., Buvens G., Karch H., Mellmann A., Caprioli A., Tozzoli R., Morabito S., Strockbine N.A., Melton-Celsa A.R., Sanchez M., Persson S., O'Brien A.D. (2012) Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing stx nomenclature. *J Clin Microbiol* **50**: 2951–63.
26. Feng P.C., Reddy S. (2013) Prevalences of Shiga toxin subtypes and selected other virulence factors among Shiga-toxigenic *Escherichia coli* strains isolated from fresh produce. *Appl Environ Microbiol* **79**: 6917–23.
27. Zweifel C., Giezendanner N., Corti S., Krause G., Beutin L., Danuser J., Stephan R. (2010) Characteristics of shiga toxin-producing *Escherichia coli* isolated from Swiss raw milk cheese within a 3-year monitoring program. *J Food Prot* **73**: 88–91.
28. Schmidt H., Maier E., Karch H., Benz R. (1996) Pore-Forming properties of the plasmid-encoded hemolysin of enterohemorrhagic *Escherichia coli* O157:H7. *Eur J Biochem* **241**: 594–601.
29. Paton A.W., Srimanote P., Woodrow M.C., Paton J.C. (2001) Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect Immun* **69**: 6999–7009.
30. Fuller C.A., Pellino C.A., Flagler M.J., Strasser J.E., Weiss A.A. (2011) Shiga Toxin subtypes display dramatic differences in potency. *Infect Immun* **79**: 1329–37.
31. Martin A., Beutin L. (2011) Characteristics of Shiga toxin-producing *Escherichia coli* from meat and milk products of different origins and association with food producing animals as main contamination sources. *Int J Food Microbiol* **146**: 99–104.
32. Melton-Celsa A.R., Darnell S.C., O'Brien A.D. (1996) Activation of Shiga-like toxins by mouse and human intestinal mucus correlates with virulence of enterohemorrhagic *Escherichia coli* O91:H21 isolates in orally infected, streptomycin-treated mice. *Infect Immun* **64**: 1569–76.
33. Bielaszewska M., Friedrich A.W., Aldick T., Schürk-Bulgrin R., Karch H. (2006) Shiga toxin activatable by intestinal mucus in *Escherichia coli* isolated from humans: predictor for a severe clinical outcome. *Clin Infect Dis* **43**: 1160–7.
34. Newton H.J., Sloan J., Bulach D.M., Seemann T., Allison C.C., Tauschek M., Robins-Browne R.M., Paton J.C., Whittam T.S., Paton A.W., Hartland E.L. (2009) Shiga toxin-producing *Escherichia coli* strains negative for locus of enterocyte effacement. *Emerg Infect Dis* **15**: 372–80.

35. Lee G.Y., Jang H.I., Hwang I.G., Rhee M.S. (2009) Prevalence and classification of pathogenic *Escherichia coli* isolated from fresh beef, poultry, and pork in Korea. *Int J Food Microbiol* **134**: 196–200.
36. Leung P.H.M., Peiris J.S.M., Ng W.W.S., Robins-Browne R.M., Bettelheim K.A., Yam W.C. (2003) A newly discovered verotoxin variant, VT2g, produced by bovine verocytotoxigenic *Escherichia coli*. *Appl Environ Microbiol* **69**: 7549–53.
37. Sanchez S., Beristain X., Martinez R., Garcia A., Martin C., Vidal D., Diaz-Sanchez S., Rey J., Alonso J.M., Herrera-Leon S. (2012) Subtilase cytotoxin encoding genes are present in human, sheep and deer intimin-negative, Shiga toxin-producing *Escherichia coli* O128:H2. *Vet Microbiol* **159**: 531–5.
38. Prager R., Fruth A., Busch U., Tietze E. (2011) Comparative analysis of virulence genes, genetic diversity, and phylogeny of Shiga toxin 2 g and heat-stable enterotoxin STIa encoding *Escherichia coli* isolates from humans, animals, and environmental sources. *Int J Med Microbiol* **301**: 181–91.
39. Wang H., Paton J.C., Paton A.W. (2007) Pathologic changes in mice induced by subtilase cytotoxin, a potent new *Escherichia coli* AB5 toxin that targets the endoplasmic reticulum. *J Infect Dis* **196**: 1093–101.
40. Amaral M.M., Sacerdoti F., Jancic C., Repetto H.A., Paton A.W., Paton J.C., Ibarra C. (2013) Action of shiga toxin type-2 and subtilase cytotoxin on human microvascular endothelial cells. *PLoS ONE* **8**: e70431.
41. Gerhardt E., Masso M., Paton A.W., Paton J.C., Zotta E., Ibarra C. (2013) Inhibition of water absorption and selective damage to human colonic mucosa are induced by subtilase cytotoxin produced by *Escherichia coli* O113:H21. *Infect Immun* **81**: 2931–7.
42. Rhoades J.R., Duffy G., Koutsoumanis K. (2009) Prevalence and concentration of verocytotoxigenic *Escherichia coli*, *Salmonella enterica* and *Listeria monocytogenes* in the beef production chain: a review. *Food Microbiol* **26**: 357–76.
43. Slanec T., Fruth A., Creuzburg K., Schmidt H. (2009) Molecular analysis of virulence profiles and Shiga toxin genes in food-borne Shiga toxin-producing *Escherichia coli*. *Appl Environ Microbiol* **75**: 6187–97.
44. Hara-Kudo Y., Konuma H., Kamata Y., Miyahara M., Takatori K., Onoue Y., Sugita-Konishi Y., Ohnishi T. (2012) Prevalence of the main food-borne pathogens in retail food under the national food surveillance system in Japan. *Food Addit Contam A* **30**: 1450–8.
45. Bettelheim K.A. (2007) The non-O157 shiga-toxigenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. *Crit Rev Microbiol* **33**: 67–87.
46. Miko A., Pries K., Haby S., Steege K., Albrecht N., Krause G., Beutin L. (2009) Assessment of shiga toxin-producing *Escherichia coli* isolates from wildlife meat as potential pathogens for humans. *Appl Environ Microbiol* **75**: 6462–70.
47. Kumar A., Taneja N., Kumar Y., Sharma M. (2012) Detection of Shiga toxin variants among Shiga toxin-forming *Escherichia coli* isolates from animal stool, meat and human stool samples in India. *J Appl Microbiol* **113**: 1208–16.
48. Zhang W., Bielaszewska M., Friedrich A.W., Kuczius T., Karch H. (2005) Transcriptional analysis of genes encoding shiga toxin 2 and its variants in *Escherichia coli*. *Appl Environ Microbiol* **71**: 558–61.